PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
A61K 35/12, 35/14, 35/28, 39/395, 39/44, C07K 14/435, 14/705, 16/18, 16/28

A1

(11) International Publication Number: WO 96/11012

(43) International Publication Date: 18 April 1996 (18.04.96)

(21) International Application Number: PCT/US95/13736

(22) International Filing Date: 10 October 1995 (10.10.95)

(30) Priority Data:
08/321,400 11 October 1994 (11.10.94) US

(71) Applicant: UNIVERSITY OF SOUTH FLORIDA [US/US]; 4202 E. Fowler Avenue FAO 126, Tampa, FL 33620-7900 (US).

(72) Inventor: SACKSTEIN, Robert; 12902 Magnolia Drive, Tampa, FL 33612 (US).

(74) Agent: KOHN, Kenneth, I.; Kohn & Associates, Suite 410, 30500 Northwestern Highway, Farmington Hills, MI 48334 (US).

(81) Designated States: CA, JP, MX, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: HEMATOPOIETIC CELL L-SELECTIN LIGAND (HLL) AND THERAPEUTICS THEREOF

(57) Abstract

An isolated and purified glycoprotein and functional analogs are disclosed. The glycoproteins are characterized by being expressed on at least primitive hematopoietic cells, and being a ligand for L-selectin. The binding of ligand to L-selectin is not inhibitied by anti-CD34 antibodies nor by MECA 79 monoclonal antibody.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

HEMATOPOIETIC CELL L-SELECTIN LIGAND (HLL) AND THERAPEUTICS THEREOF

BACKGROUND OF THE INVENTION

TECHNICAL FIELD

The present invention involves the development of compounds which can regulate and control the function of adhesion molecules.

BACKGROUND ART

The adhesion molecules are involved in the fundamental control of cell-cell interaction and cellular migration. Adhesion molecules regulate diverse processes in inflammation, hematopoiesis and tumor metastasis. (Woodruff, et al, 1987; Springer, et al, 1987; Sharon and Lis, 1993; Scakstein, 1993) For general reviews on adhesion molecules see Carlos and Harlan, 1994 and Chin et al, 1991. It would be useful to develop reagents which can control and regulate the adhesion proteins, particularly within the selectin family.

The peripheral lymph node "homing receptor", L-selectin (CD62L), is a ~75 kDa glycoprotein which mediates attachment of lymphocytes to lymph node (LN) high endothelial venules (HEV), an adhesive interaction which is the first step in the migration of lymphocytes from blood into lymphoid tissues (Gowans and Knight, 1964; Marchesi and Gowans, 1964). This trafficking of lymphocytes from blood into lymph nodes is markedly nonrandom and is initiated by specific adherence of the lymphocytes to HEV. The "lymph node homing receptor" or L-selectin (LECAM-1) is the principal lymphocyte membrane glycoprotein mediating this attachment.

The L-selectin protein is recognized by a variety of monoclonal antibodies (mAbs) in humans {Gatenby et al., 1982 (Leu-8); Reinherz et al., 1982 (TQ-1); Tedder et al., 1990 (LAM)} and is a member of the selectin family of adhesion molecules, which includes P-selectin (CD62P) and E-selectin (CD62E). Selectins share a common structure consisting of an amino-terminal calcium-dependent lectin domain, an epidermal growth factor domain, a variable number of repeat sequences bearing homology to complement regulatory and catalytic proteins binding C3b or C4b, a transmembrane portion, and a C-terminal cytoplasmic

tail (Bevilacqua and Nelson, 1993; Rosen, 1993). The molecular weight varies among leukocytes due to posttranslational glycosylation among subsets of leukocytes.(Carlos and Harlan, 1994) The lectin domain of these proteins directs their adhesion to carbohydrate molecules present on the cell surface.

The adhesive interaction between lymphocytes and HEV has been extensively analyzed using an *in vitro* binding assay (Stamper and Woodruff, 1976). This assay is performed under shear at 4°C, whereby binding mediated by L-selectin is maximized and effects of other adhesion molecules are minimized (Shaw et al, 1986; Spertini et al., 1991). The interaction of L-selectin with its corresponding ligand(s) on HEV is calcium-dependent (Woodruff et al., 1977) and requires the presence of sialic acid (Rosen et al., 1985; True et al., 1990) and sulfate (Imai et al., 1993) on the ligand(s). L-selectin behaves as a lectin and recognizes sialylated, high mannose residues on its corresponding ligand which is identified by the monoclonal antibody MECA-79 (Sackstein, 1993). MECA-79 identifies an L-selectin ligand on lymph node HEV and which cross-reacts with GLYCAM-1 and CD34. *In vitro* adherence of lymphocytes via L-selectin can be inhibited by carbohydrates such as mannose-6-phosphate (man-6-P), PPME (Phosphomannan

monoester core from *Hansenula hostii*, a phosphomannosyl-rich polysaccharide), and fucoidin (a sulfated, fucose-rich polysaccharide) (Stoolman and Rosen, 1983).

Ligands for L-selectin have thus far been characterized on murine endothelial cells utilizing a murine L-selectin IgG chimera molecule as a probe. (Watson et al., 1990) This approach has identified two proteins, GlyCAM-1 (Sgp50) (Imai et al., 1991) and CD34 (Sgp90) (Baumhueter et al., 1993), present on endothelial cells. GlyCAM-1 is a novel sialomucin, and its role as a ligand for L-selectin is its only known function (Lasky et al., 1992). Although present on endothelial cells in most tissues (Beschorner et al., 1985), CD34 is best known for its expression on the earliest multilineage colony-forming hematopoietic stem cells (Civin et al., 1984).

Hematopoietic progenitor cells characteristically express both L-selectin and CD34 (Terstappen et al., 1992), and there is growing evidence that L-selectin plays a role in hematopoiesis (Terstappen et al., 1993; Kobayashi et al., 1994). The characterization of L-selectin and its ligands among progenitor cells is of considerable interest as adhesion proteins regulate

cell-cell and cell-stromal interactions fundamental to hematopoiesis.

In general assays for determining the adhesion between lymphocyte and HEV requires the use of frozen sections of lymph nodes. (Stamper and Woodruff, 1976; Sackstein et al, 1988) It would be useful to be able to use cells in suspension in the assay also. This would enable the use of cell lines, giving rise to more reproducible results as well as reducing the need for surgical procedures for lymph node removal.

It would be useful to have strategies which would allow regulation of hematopoiesis since it is regulated by cell-cell and cell-stromal interactions. For example, Terstappen et al (1993) have shown that activation of L-selectin increases the clonogenic capacitiy of stem cells.

During recovery of immune function following bone marrow transplantation pathologic changes have been observed following transplantation which interfer with lymphocyte migration and HEV integrity. Further, in addition to changes in lymph node structure, alterations in lymphocyte migration can occur secondary to the effect of pharmacologic agents used in posttransplant therapy such as corticosteroids (Sackstein, 1993). It would be useful to have an

agent which can assist in reestablishing lymphocyte trafficking and so immune function following bone marrow transplantation.

The crucial role of adhesion molecules in controlling and directing the inflammatory process indicates that a reagent which interferes with the process, i.e. anti-adhesive, could have anti-inflammatory properties.

Further, cell adhesion molecules are involved in metastasis, therefore it would be useful to develop an anti-adhesive which has anti-metastatic properties. In particular, with the identification of L-selectin on hematopoietic cells, it would be useful to have an anti-adhesive that affects L-selectin in leukemia to decrease the growth and spread of malignant hematopoietic cells throughout the body.

Further, it would be useful to have additional cell markers and monoclonal antibodies directed against these cell markers to allow for cell targeting.

SUMMARY OF THE INVENTION AND ADVANTAGES

According to the present invention, an isolated and purified glycoprotein and functional analogs are disclosed. The glycoproteins are characterized by being expressed on at least primative hematopoietic cells, and being a ligand for L-selectin. The binding of ligand to L-selectin is not inhibited by anti-CD34 antibodies nor by MECA-79 monoclonal antibody.

BRIEF DESCRIPTION OF THE DRAWINGS

Other advantages of the present invention will be readily appreciated as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings wherein:

FIGURE 1A-B are photomicrographs of cytospin preparations of KG1a cells demonstrating adherence of lymphocytes (small dark dots), (A) Lymphocytes adhere to KG1a in the presence of CD45 or isotype control Abs, (B) Lymphocyte binding assay in the presence of LAM1-3 Ab (anti-L-selectin);

FIGURE 2 is FACS profiles of lymphocytes used in the binding assay after incubation with isotope-matched IgG control, LAM1-3, or anti-CD45 Abs, followed by GAM-FITC, results shown are representative of 3 independent experiments;

FIGURE 3A-D are FACS profiles of KG1a cells sorted by FACS prior to the binding assay into CD34+ and CD34- fractions using mAb HPCA-2PE, sorted cell fractions were restained for CD34 using mAb QBEND10-FITC and analyzed, positive and negative sorted fractions were >90% and <10% positive for CD34, respectively, results shown are representative of 3 independent experiments;

FIGURE 4 is a photomicrograph showing the lymphocyte adherence assay performed on the sorted cells, and no differences in lymphocyte adherence were evident among the CD34+ and CD34- populations, adherence to the CD34 negative fraction is shown; and

FIGURE 5A-F are FACS profiles of COS-7 cells were transfected with either CD34-pCDM8 (E,F) or pCDM8 (mock, C,D), then analyzed by FACS and compared to KG1a (A,B) for CD34 expression, Abs used were isotype-matched IgG_1 control and anti-CD34 mAb QBEND10, Lymphocytes did not adhere to CD34-transfected COS-7 cells, despite higher levels of CD34 expression as compared to KG1a cells.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention provides an isolated and purified glycoprotein and functional analogs thereof. Analog is defined as a molecule that will be generally at least 70% homologous over any portion that is functionally relevant. In more preferred embodiments the homology will be at least 80% and can approach 95% homology to the amino acid sequence of the protein segment of the glycoprotein. The homology will extend over a region of at least 8 contiguous amino acids to 80 contiguous amino acids. The amino acid sequence of an analog may differ from that of the glycoprotein of the present invention when at least one residue is deleted, inserted or substituted. The molecular weight of the glycoprotein may vary between the analog and the present invention due to carbohydrate differences. Differences in glycosylation may be present between the analog and the present invention.

The glycoprotein has the following functional characteristics. It is expressed on at least primative hematopoietic cells. The glycoprotein is a ligand for L-selectin. The ligand binding to L-selectin is not inhibited by anti-CD34 antibodies and is not recognized by the

MECA-79 monoclonal antibody. The glycoprotein is designated hereinafter as hematopoietic cell L-selectin ligand, HLL.

Further, the glycoprotein, HLL, is a membrane associated glycoprotein and functions as an adhesion protein ligand. The glycoprotein facilitates attachment of lymphocytes to hematopoietic cells including primitive hematopoietic cells.

The present invention also provides for an antibody directed against the glycoprotein, HLL. The antibodies may be either monoclonal or polyclonal. Murine monoclonal antibodies are initially raised against KG1a cells. The monoclonals that are generated are then screened for the ability to block lymphocyte binding to KG1a.

Utilizing these monoclonal antibodies, the glycoprotein is isolated by immunoprecipitation of KG1a membrane lysates as is standard in the art and used for the production of further antibodies as needed. Such methods can be found described Sambrook et al, *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor, New York, 1989, as well as additional methods of isolation and purification as are known in the art.

Additionally, the antibodies may be prepared against a synthetic peptide based on the sequence, or prepared recombinantly by cloning techniques or the natural gene product and/or portions thereof may be isolated and used as the immunogen. Such proteins or peptides can be used to produce antibodies by standard antibody production technology well known to those skilled in the art as described generally in Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988.

For producing polyclonal antibodies a host, such as a rabbit or goat, is immunized with the protein or peptide, generally with an adjuvant and, if necessary, coupled to a carrier; antibodies to the protein are collected from the sera.

For producing monoclonal antibodies the technique involves hyperimmunization of an appropriate donor with the protein or peptide fragment, generally a mouse, and isolation of splenic antibody producing cells. These cells are fused to a cell having immortality, such as a myeloma cell, to provide a fused cell hybrid which has immortality and secretes the required antibody. The cells are then cultured, in bulk, and the monoclonal antibodies harvested from the culture media for use.

The antibody can be bound to a solid support substrate or conjugated with a detectable moiety or be both bound and conjugated as is well known in the art. (For a general discussion of conjugation of fluorescent or enzymatic moieties see Johnstone & Thorpe, *Immunochemistry in Practice*, Blackwell Scientific Publications, Oxford, 1982.) The binding of antibodies to a solid support substrate is also well known in the art. (see for a general discussion Harlow & Lane *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Publications, New York, 1988) The detectable moieties contemplated with the present invention can include, but are not limited to, fluorescent, metallic, enzymatic and radioactive markers such as biotin, gold, ferritin, alkaline phosphatase, ß-galactosidase, peroxidase, urease, fluorescein, rhodamine, tritium, ^{14}C and iodination.

The method of targeting cells includes the steps of preparing antibodies directed against the glycoprotein as described above and coupling the antibodies to the appropriate agent whether for cell killing, cell selection or cell identification. For cell killing, toxins such as ricin A chain, pseudomonas exotoxin A, diphtheria toxin, other plant and bacterial toxins as well as

chemotherapeutic compounds can be coupled in the present invention forming an immunotoxin. For a general review of the antibody-toxin art see Ramakrishnan, 1990.

Cell targeting requires exposing a population of cells to the immunotoxin. A toxin bound antibody can be administered to the appropriate patient and targeted cells killed *in vivo*. The immunotoxin is administered and dosed in accordance with good medical practice, taking into account the clinical condition of the individual patient, the site and method of administration, scheduling of administration, and other factors known to medical practitioners. The "effective amount" for purposes herein is thus determined by such considerations as are known in the art. The amount must be effective to achieve at least 25% of the treated patients exhibit improvement including but not limited to improved survival rate, more rapid recovery, or improvement or elimination of symptoms.

Alternatively, cells can be removed from the patient and treated *ex vivo* selectively. For example, cells expressing HLL can be removed through complement-mediated lysis from the *ex vivo* population and the remaining cells returned to the patient. Additional cell removal can be undertaken

utilizing cell sorting, "panning" and magnetic bead separation. Further, utilizing cell sorting, "panning", magnetic bead separation and the like cell populations can be enriched for HLL bearing cells and this enriched cell population returned to the patient.

The targeted cells to be removed are cells expressing HLL and can be selected from the group consisting of leukemic cells, malignant hemopoietic progenitor cells, or any malignant cell expressing the marker.

The present invention also provides a method of regulating hematopoiesis, particularly in reconstitution of the immune system following bone marrow transplantation. The present invention includes the steps of selecting those cells with high(+) or low(-) expression of HLL depending on the growth characteristics associated with the marker density needed by the patient. The selection procedure utilizes *ex vivo* methods as described herein. After selection, the selected cell type is cultured *in vitro*, if needed to expand the population using standard methods known in the art. The patient is then infused with the expanded, enriched HLL+ or HLL- population as needed.

The present invention further provides a method of regulating inflammatory response by interrupting cellular migration into lymph nodes and sites of both acute and chronic inflammation including the step of administering to the patient antibody directed agains HLL thereby disturbing cellular migration mediated by HLL by blocking the the lymphocyte attachment site and can be injected directly at the inflammed site if needed. The regulation of the inflammatory response would be useful in autoimmune disorders, post-ischemic tissue injury and sepsis (Calos and Harlan, 1994). Administration and effective dose are as described for immunotoxins hereinabove.

Studies by applicant on the interaction of L-selectin and hematopoietic CD34 function as an adhesive receptor-ligand pair, *in vitro* binding studies of lymphocytes to KG1a, a primitive CD34-positive human cell line derived from an acute myeloid leukemia (Civin et al., 1994; Koeffler et al., 1980) lead to the present invention. These studies surprisingly revealed highly specific adherence of lymphocytes to KG1a cells mediated by L-selectin on the lymphocyte, but unexpectedly not involving CD34 as the corresponding ligand as had been previously reported (Baumhueter et al, 1993; Oxley and Sackstein, 1994). The results indicated

the presence of a ligand, designated HLL, for L-selectin on the surface of this hematopoietic progenitor cell line and provide the first evidence of L-selectin-mediated adhesion between lymphocytes and a non-endothelial cell type.

For these studies, the lymphocyte-HEV adherence assay was used which is an *in vitro* approximation of physiologic adhesion mediated by L-selectin was used. It has been a fundamental tool in studying the function of L-selectin in its native state on the surface of lymphocytes. This binding assay was novelly adapted to examine lymphocyte-hematopoietic cell adhesion, and the results provide the unexpected results of L-selectin-dependent adhesive interactions between lymphocytes and non-endothelial cells.

The adaptation of the assay allowed for the first time the use of cell lines in a lymphocyte-HEV adherence assay. In this assay, slides were prepared of KG1a cell suspensions which were used in place of slides of frozen lymph node sections as taught by the prior art. The KG1a cells were placed on the slides by using cyto-spin centrifugation as further described hereinbelow.

Several independent lines of evidence indicate that lymphocyte binding to KG1a is mediated primarily, if not solely, by L-selectin.

First, an anti-L-selectin mAb (LAM1-3) previously shown to block L-selectin-mediated adherence to LN HEV (Spertini et al., 1991), completely inhibited PBL from binding to KG1a or LN HEV, whereas anti-CD45 and isotype control Abs did not block lymphocyte binding. Second, L-selectin-mediated binding is a calcium-dependent event, and lymphocytes were unable to bind to KG1a in the presence of the calcium chelator EDTA. Third, carbohydrates such as man-6-P, PPME, and fucoidin inhibited lymphocyte adherence to KG1a. These compounds are all known to bind to L-selectin and to inhibit lymphocyte binding to HEV in the in vitro assay (Stoolman and Rosen, 1983; Stoolman et al., 1984). Lastly, it is known that PMA treatment of lymphocytes causes shedding of membrane L-selectin via a protein kinase C activation pathway, and corresponds to the loss of lymphocyte binding to LN HEV in the *in vitro* assay (Tedder et al., 1990). In these studies, PMA-treated PBL were no longer able to bind to KG1a.

The nature of the ligand was investigated by determining the effects of various enzyme treatments of KG1a on the binding capacity. Previous studies have shown that ligand expression of sialic acid is essential for L-selectin-mediated binding of lymphocytes to LN HEV (Rosen et al.,

1985). In the present examples, neuraminidase-treated KG1a showed a complete loss of lymphocyte binding, indicating that sialic acid residues are also a necessary component on the KG1a L-selectin ligand; as such, lymphocyte adherence to KG1a involves carbohydrate motifs and is not based strictly on protein-protein interactions. This finding, combined with the results of protease experiments, indicates that the KG1a ligand is a glycoprotein.

To examine whether O-linked glycosylations on the ligand play a central role in the adhesive interaction, KG1a were digested with the enzyme O-sialoglycoprotein endopeptidase which specifically cleaves proteins at sites of O-linked sialo-glycosylation (Abdullah et al., 1992) and which has been shown to differentially cleave epitopes of the CD34 molecule (Sutherland et al., 1992). The data reveal that treatment of KG1a in suspension with the enzyme actively destroyed CD34 epitopes, yet had no effect on lymphocyte adherence. These results suggest that ligand sialic acid residues critical to binding are present on N-linked rather than on O-linked glycosylations.

CD34 has been reported to be a ligand for L-selectin based on the finding that a murine L-

selectin-IgG chimera molecule precipitated CD34 from a murine lymph node lysate (Baumhueter et al., 1993). The results as set forth in the examples indicate that CD34 as expressed on KG1a is not a functional ligand for lymphocyte L-selectin, as no difference in lymphocyte binding to sorted CD34- and CD34+ KG1a cells was observed. (Figures 3 and 4) Titration studies using varying proportions of KG1a and HL60 have demonstrated that the amount of lymphocyte adherence is directly proportional to the percentage of input KG1a cells, indicating that differences in lymphocyte binding to the positive and negative sorted fractions would have been evident if CD34 were the ligand. It is unlikely that a particular binding epitope of CD34 as selected, as this experiment was done using two different anti-CD34 mAbs to sort the KG1a. Two forms of CD34 on KG1a have been reported ("truncated" and "full length") (Krause et al., 1993); however, these differences do not account for the data here as sorting was also performed using QBEND10, which recognizes both forms.

In addition to sorting experiments, evidence that CD34 is not the L-selectin ligand on KG1a is derived from mAb blocking studies and adherence assays using other CD34 positive cells. None of the anti-CD34 mAbs tested, or any

combination thereof, was able to block lymphocyte binding to KG1a. Furthermore, lymphocytes did not adhere to another primitive CD34+ cell line, RPMI 8402, and transfection of CD34 into COS-7 cells did not confer lymphocyte binding capacity. While potential glycosylation differences of the CD34 molecule expressed by these cell types could affect their ability to support lymphocyte adherence, this explanation is unlikely in light of equivalent adherence observed among the sorted CD34+ and CD34- KG1a cells. Taken together, the data presented here indicate that the CD34 glycoform present on hematopoietic cells is not a ligand for L-selectin. Moreover, flow cytometric analysis of the various cell lines utilized in the binding assay provides evidence that membrane structures such as LFA-1, VLA-4, CD44, Sialyl LeX and CD43 do not play a primary role in lymphocyte adherence to KG1a since each of these molecules were also present on at least one other cell line tested that did not demonstrate lymphocyte binding.

HLL is not recognized by MECA-79 monoclonal antibody which identifies L-selectin ligands on lymph node HEV. Immunofluorescence analysis of KG1a using MECA-79 shows no evidence of the protein identified by MECA-79. HLL is shown to

be unique from L-selectin ligands thus far identified.

In the present study, direct cell-cell interactions were utilized to detect the presence of an L-selectin ligand on a hematopoietic cell. Other studies directed at identifying L-selectin ligands have relied on molecular approaches utilizing a murine L-selectin-IgG chimera molecule, synthesized in a human embryonal kidney cell line, as a probe (Watson et al., 1990). Of note, studies utilizing this chimera have failed to demonstrate binding of the molecule to KG1a cells (Majdic et al., 1994). In general, tissue- and species-specific patterns of glycosylations are well described, (Yamashita et al., 1983; Cullen et al., 1981; Yamashita et al., 1985) and such differences can affect the biological activity of proteins expressed in different cells (Cowing 1983; Huff et al., 1983). As it is known that glycosylation of L-selectin varies among different cells expressing the protein (Lewinsohn et al., 1987, Ord et al., 1990; Griffin et al., 1990), such differences may account for the observation here that native L-selectin, expressed on lymphocyte membranes, selectively binds to a corresponding ligand on KG1a cells while the chimera apparently does not. Similarly, differences in glycosylation of CD34

among endothelial cells and hematopoietic cells may account for the differential capacity of this protein to participate in L-selectin interactions among these cell types.

L-selectin ligands have been recognized heretofore only on endothelial cells. The detection of an L-selectin ligand on a non-endothelial cell expands the physiologic implications of L-selectin function beyond its well-characterized role in regulating leukocyte trafficking.

The above discussion provides a factual basis for the characterization and use of HLL. The methods used with and the utility of the present invention can be shown by the following examples.

EXAMPLES

GENERAL METHODS:

Cell Lines. Cell lines used in these studies were obtained from the following sources: KG1a and Nalm 16, gift of Dr. William E. Janssen; HL60, K562, and Raji, gift of Dr. Lynn Moscinski; COS-7, gift of Dr. Kenneth Zukerman (all from H. Lee Moffitt Cancer Center, Tampa, FL); RPMI 8402, gift of Dr. Daniel G. Tenen (Harvard Medical School, Boston, MA). All cells were cultured in RPMI 1640 (Gibco-BRL, Gaithersburg, MD)

supplemented with 10% heat-inactivated fetal bovine serum (FBS) in a humidified chamber at 37°C with 5% CO_2 in air.

Preparation of Lymphocytes. Human peripheral blood lymphocytes (PBL) were isolated by Ficoll density gradient from blood drawn in sodium citrate. To obtain rat thoracic duct lymphocytes (TDL), thoracic ducts of rats were cannulated as described by Bollman et al.(1948). Lymph was collected in phosphate buffered saline (PBS) with 0.1% penicillin/streptomycin and 5 U/ml heparin. PBL or TDL were washed three times in RPMI 1640 medium without bicarbonate (Gibco-BRL), pH 7.4, and suspended at 1 x 10^7 cells/ml in above medium with 5% FBS and kept on ice until use in the adherence assay.

Lymphocyte Adherence Assay. The procedure for the *in vitro* binding of human or rat lymphocytes to KG1a was adapted from the rat lymphocyte-lymph note binding assay which has been described by Stamper and Woodruff (1976) and Sackstein et al.(1988). Cytospin preparations of KG1a or other cell lines were made on a Cytospin 3 Cytocentrifuge (Shandon Lipshaw, Pittsburgh, PA). Frozen rat LN sections 8μm thick were mounted on slides, and lymphocyte binding to LN HEV served as a positive control in all experiments. Slides were

air dried, fixed in 3% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA) in PBS, rinsed with PBS, incubated in 0.2M L-lysine (Sigma Chemical Company, St. Louis, MO) to block unreacted glutaraldehyde, then rinsed and held in RPMI 1640 with 1% FBS at 4°C until use in experiments.

Lymphocyte suspensions (200µl) were overlaid onto cytospin or LN sections in duplicate and placed on a rotating platform (80rpm) at 4°C for 30 minutes. Slides were then rinsed in cold PBS to remove non-adherent lymphocytes, fixed in 3% glutaraldehyde, and stained with methyl green-thionin. Slides were examined under the light microscope for adherence of lymphocytes to KG1a or LN HEV.

Number of lymphocytes adherent to confluent area of KG1a were counted by light microscopy using an ocular grid under 250X magnification. Quantitation was performed by examining two fields per slide, minimum of two slides per experiment, three separate experiments. Results are presented as % binding compared to corresponding untreated control sections.

Treatment of Lymphocytes with Potential Inhibitors. Lymphocytes in RPMI 1640 medium with 5% FBS were pre-incubated (30 min on ice) and the assay performed in the presence of the following

inhibitors: 1mM EDTA (no pre-incubation period); 10 mM D-mannose-6-phosphate (Sigma); 10 μg/ml PPME (kindly provided by Dr. M.E. Slodki, USDA, Peoria, IL); and 5 μg/ml fucoidin (Sigma).

Antibody Blocking Experiments. Lymphocytes (1 x 10^7 cells/ml) were pre-incubated on ice for 20 minutes with mAbs at 1.0 μg/ml and used in the binding assay without further washing. The following mAbs were used: LAM1-3 (anti-L-selectin) (kind gift of Dr. Thomas Tedder, Duke University, Durham, N.C., and also obtained from Coulter Corp., Hialeah, FL); anti-CD45 (leukocyte Common Antigen) (Becton Dickinson, San Jose, CA); and IgG_1 (isotype control) (Coulter). In some experiments, prepared KG1a slides were incubated with 0.2 μg of anti-CD34 Abs {HCPAa-1 (clone My10) and HPCA-2 (clone 8G12) (Becton Dickinson), QBEND10 (AMAC) and 12.8 (kindly provided by Dr. Pat Roth, Coulter Corp.)} in RPMI 1640 with 5% FBS for 30 minutes prior to the binding assay.

PMA Treatment of Lymphocytes. Lymphocytes were suspended at 1 X 10^7 cells/ml in cell culture medium and incubated 1 hour at 37°C with or without 10 ng/ml PMA (Gibco-BRL). Cells were then washed twice in PBS and used in either the lymphocyte binding assay or analyzed for surface antigens by flow cytometry (see below).

Enzyme Treatment of KG1a or LN. Cytospin preps KG1a or LN frozen sections were glutaraldehyde-fixed, then treated with various enzymes prior to the binding assay. For treatment with neuraminidase (sialidase), slides were rinsed twice with enzyme buffer (50 mM NaAc, 154 mM NaCl, 9 mM $CaCl_2$, pH 5.5), then incubated 30 min. at 37°C with 50 µl of buffer (control) or undiluted neuraminidase (1.2 U/ml, Boehringer Mannheim, Indianapolis, IN). In protease studies, slides were incubated with RPMI 1640 alone or RPMI 1640 containing enzymes: 100 U/ml chymotrypsin (Sigma) (115 min. at 37°C), or 0.1% bromelain (Sigma) (30 min. at 37°C); to assess specificity, the protease inhibitors PMSF (1.0 mg/ml, Sigma) and chymostatin (900 µg/ml, Boehringer Mannheim) were coincubated with chymotrypsin (100 U/ml) for 15 min. at 37°C. Following enzyme treatments, slides were washed X3 with RPMI 1640 and placed in RPMI 1640 with 1% FBS until use in the binding assay.

KG1a cells in suspension (4 x 10^7 cells/ml) were incubated with O-sialoglycoprotein endopeptidase (Accurate Chemical and Scientific Corp., Westbury, NY) (0.24 mg/ml, 37° C, 30 min.), washed X3 with 2% FBS in PBS, and cytospin preparations were made for use in the binding assay. To verify the activity of the enzyme, cells

were tested for the cleavage of CD34 by flow cytometry using QBEND10 mAb.

Antigen Expression by Flow Cytometry. Flow cytometric analysis was performed using the following commercially-available mAbs together with isotype-matched controls: TQ1 (anti-L-selectin), LAM1-3 (Anti-L-selectin), 4B4 (Anti-VLA-4) (all from Coulter Corp.); QBEND10 (anti-CD34) (AMAC, Westbrook, ME); anti-CD44, LFA-1-β (anti-CD18), LFA-1-α (anti-CD11a), HPCAS-2 (anti-CD34), anti-CD45, Leukosialin (anti-CD43), anti-Sialyl-Le^x (all from Becton Dickinson). Cells (1 X 10^6) in 100 μl of PBS with 2% FBS were incubated on ice for 25 minutes with Ab as per manufacturer's recommendations, washed X3 and analyzed on a FACStar^{PLUS} (Becton Dickinson).

Fluorescence Activated Cell Sorting of KG1a cells. KG1a cells were stained with anti-CD34 mAbs (QBEND10-FITC in two experiments, HPCA-2-PE in one experiment) and positive and negative expressing cells were sorted on a FACstar^{PLUS} flow cytometer equipped with an argon laser tuned at 488 nm (Becton Dickinson). Sorted cell populations were restained with anti-CD34 Ab directed at epitopes not used for sorting and were analyzed to determine the efficiency of the sort. Cytospin preparations were made of the positive and negative

sorted fractions and were used in the lymphocyte binding assay.

Transfection of COS-7 with CD34 cDNA. COS-7 cells were transiently transfected with human full-length CD34 cDNA in pCDM8 plasmid (a gift from Dr. Daniel Tenen, Boston, MA) using a DEAE Dextran transfection method (Selden, 1992). Briefly, COS-7 cells were incubated for 4 hours at 37°C with 10 ml of transfection solution containing 20-40 μg of plasmid DNA, 10% Nu Serum (Collaborative Biomedical Products, Bedford, MA), 400 μg/ml DEAE Dextran (Sigma), and 100 μ chloroquine (Sigma) in Dulbecco's Modified Eagles Medium (Gibcon-BRL). Cells were then rinsed and treated with 10% DMSO (Sigma) in PBS for two minutes at room temperature, rinsed in PBS, and incubated in tissue culture media for 3 days. In one set of experiments, trypsinization was avoided by growing transfected cells directly on glass slides for subsequent use in the binding assay or for analysis of CD34 expression by fluorescence microscopy. In other experiments, COS-7 cells grown on 10 cm plates were removed with trypsin/EDTA (0.25%/1mM, Gibco-BRL), then analyzed for CD34 expression by flow cytometry. These trypsinized cells were then placed on slides by cytospin for use in the lymphocyte binding assay.

EXAMPLE 1

Lymphocytes Bind to KG1a. Lymphocytes (both PBL and TDL) adhered specifically and reproducibly to KG1a, but not to RPMI 8402, HL60, Nalm 16, K562, or Raji cell lines in the *in vitro* binding assay (Table 1). All experiments were performed in parallel with LN frozen sections as positive controls. Lymphocyte binding to KG1a was observed under conditions identical to those whereby L-selectin mediates binding of lymphocytes to LN HEV.

Lymphocyte Binding to KG1a is Mediated by L-selectin. To directly examine whether lymphocyte attachment was mediated by L-selectin, PBL were pre-incubated with the anti-L-selectin mAb LAM1-3, anti-CD45, or IgG_1 isotype control Abs. The LAM1-3 Ab completely inhibited lymphocyte binding to KG1a and LN control, while CD45 and isotype control mAbs did not affect binding (Fig. 1A & 1B). In order to quantify the relative amounts of Ab attachment to lymphocytes, Ab-treated lymphocytes were incubated with goat-anti-mouse FITC-conjugated secondary Ab and analyzed by flow cytometry. Although the amount of anti-CD45 Ab on lymphocytes was significantly greater than that of LAM1-3 as indicated by mean channel fluorescence (Fig. 2),

LAM1-3 alone blocked lymphocyte adherence to KG1a and LN HEV, indicating that this effect was specific and not secondary to charge or steric alterations of the lymphocyte membrane.

The Effect of Enzyme Treatment of KG1a on Lymphocyte Binding. Pretreatment of both KG1a and LN control sections with neuraminidase (60 mU), chymotrypsin (100 U/ml) or bromelain (0.1%) prior to the binding assay abrogated binding of lymphocytes, while treatment with buffer or medium alone did not alter binding capacity. In addition, the effects of chymotrypsin were confirmed by coincubation with the protease inhibitors chymostatin and PMSF, which prevented chymotrypsin effects on lymphocyte binding. However, pretreatment of KG1a with O-sialoglycoprotein endopeptidase had no effect on lymphocyte binding despite complete enzymatic removal of the CD34 epitope recognized by QBEND10 mAb. (Table 2)

Lymphocyte Binding to KG1a is Calcium Dependent. Lymphocyte binding to KG1a and to LN control sections was completely inhibited by the presence of EDTA, indicating a calcium requirement for lymphocyte-KG1a binding.

Mannose-6-Phosphate, PPME, and Fucoidin Inhibit Lymphocyte Binding to KG1a. The specificity of lymphocyte-KG1a binding was investigated by treating PBL or TDL with carbohydrate inhibitors of L-selectin-HEV interactions prior to the adherence assay. Man-6-P (10 mM), PPME (10 μg/ml), and fucoidin (5 μg/ml) all inhibited lymphocyte binding to both KG1a and LN control sections. (Table 2)

PMA Treatment of Lymphocytes Results in the Loss of Binding to KG1a. PBL were incubated for 1 hour at 37°C with 10 ng/ml PMA, then used in the lymphocyte binding assay. PMA-treated PBL were unable to bind to either KG1a or LN HEV, while control PBL demonstrated high amounts of binding. (Table 2)

Loss of surface L-selectin was assessed by flow cytometric analysis of TQ1 levels in control and PMA-treated PBL. PMA-treated lymphocytes showed a dramatic decrease in TQ1 mean channel fluorescence (to levels less than 10% of that of untreated cells) in three separate experiments. PMA-treated PBL were also analyzed for expression of CD44, LFA-1 (both α and ß chains), and VLA-4, and expression of these

adhesion molecules following PMA exposure was identical to expression on control PBL.

EXAMPLE 2

Pretreatment of KG1a with Anti-CD34 Antibodies Did Not Inhibit Adherence of Lymphocytes. Cytospin preps of KG1a were preincubated with anti-CD34 Abs and the binding assay was performed in the presence of the Abs. Monoclonal ABS to four different CD34 epitopes were used alone or in combination, including the clones My10, QBEND10, 8g12, and 12.8, in amounts ranging from 0.2 to 17 μg/slide. Anti-CD45 (irrelevant control) and IgG_1 (isotype control) Abs were also tested. None of the anti-CD34 Abs inhibited lymphocyte binding to KG1a, despite immunohistochemical evidence of extensive Ab binding to the glutaraldehyde-fixed KG1a sections.

Other Surface Antigens on KG1a do not Appear to Mediate Binding. The surface expression of several antigens on KG1a, RPMI 8402, HL60, Nalm 16, K562, and Raji was analyzed by flow cytometry (Table 1). LFA-1, FLA-4, CD44, Sialyl Le^x, and CD43 were all expressed by KG1a and at least one other cell line that did not support lymphocyte adherence. Of note, although RPMI 8402 cells

express CD34 at levels comparable to KG1a, there was no adherence of lymphocytes to these cells in the binding assay.

CD34 Positive and Negative KG1a Cells Supported Equivalent Amounts of Lymphocyte Binding. CD34+ and CD34- KG1a cells were separated by fluorescence activated cell sorting and cytospin preparations of each population were made. The *in vitro* adherence of lymphocytes was identical in the CD34+ and CD34- populations despite an enrichment of >90% and <10% CD34+ cells in the respective populations (Fig. 3 and 4; Table 2).

EXAMPLE 3

CD34-Transfected COS-7 Cells Did Not Support Lymphocyte Adherence. COS-7 cells were transfected with CD34 and tested in the *in vitro* binding assay, and both trypsinized and intact transfected COS-7 cells failed to support lymphocyte adherence. By flow cytometric analysis, transfected cells were approximately 60% positive for CD34 expression, and the mean channel fluorescence was greater than that of KG1a control cells (Fig. 5). Intact, untrypsinized COS-7 cells transfected with CD34 also strongly expressed CD34

(≈90% positive as estimated by fluorescence microscopy).

Throughout this application various publications are referenced by citation or number. Full citations for the publications referenced by number are listed below. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

The invention has been described in an illustrative manner, and it is to be understood that the terminology which has been used is intended to be in the nature of words of description rather than of limitation.

Obviously, many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.

Table 1. Expression of Surface Molecules on Cell Lines Utilized in the Lymphocyte Adherence Assay.

CELL LINE	LYMPHOCYTE ADHERENCE	LINEAGE	RELATIVE EXPRESSION OF MEMBRANE PROTEINS*					
			CD34	LFA-1	VLA-4	CD44	Sialyl Le^x	CD43
KG1a	YES	Myeloid	++++	++++	++++	++++	++++	++++
RPMI 8402	NO	Lymphoid	++++	-	++++	+++	-	++++
HL60	NO	Myeloid	-	-	++++	+	-	++++
Nalm 16	NO	Lymphoid	-	-	++++	-	+++	++++
K562	NO	Erythroid	-	-	-	-	++	+++
Raji	NO	Lymphoid	-	+++	++++	-	-	+

*Percentage of positive cells as determined by flow cytometric analysis.
- = 0-5% positive
+ = 6-35% postive
++ = 36-65% positive
+++ = 66-95% positive
++++ = >96% positive

Table 2. Lymphocyte Adherence to KG1a

LYMPHOCYTE TREATMENT	Mean (SEM) of Binding (% of Untreated Control)
EDTA	0.3 (0.3)
Mannose-6-P	5.7 (1.0)
Fucoidin	1.4 (0.4)
PPME	5.4 (0.5)
LAM1-3 mAb	1.9 (0.4)
Anti-CD45 mAb	98.7 (6.3)
IgG_1 Control mAb	115.1 (9.0)
PMA	1.1 (0.3)
KG1A TREATMENT:	
Anti CD34 mABs†	116.2 (7.7)
Anti CD45 mAb	98.0 (3.6)
IgG_1 Control mAb	101.8 (8.5)
CD34-Positive Sort	102.8 (3.5)
CD34-Negative Sort	104.1 (4.2)
Neuraminidase	3.1 (0.7)
Neuraminidase Buffer Control	100.5 (6.7)
O-Sialoglycoprotein Endopeptidase	98.4 (2.3)
Bromelain	3.8 (0.4)
Chymotrypsin	6.7 (0.7)
Chymotrypsin, PMSF, Chymostatin	94.0 (3.8)

†Combination of HPCA-1, HPCA-2, 12.8 and QBEND10 mAbs.

REFERENCES

Abdullah KM, Udoh EA, Shewen PE, Mellors A: A neutral glycoprotease of *Pasteurella haemolytica* A1 specifically cleaves O-sialoglycoproteins. Infect Immun 60:56, 1992.

Baumhueter S, Singer MS, Henzel W, Hemmerich S, Renz M, Rosen SD, Lasky LA: Binding of L-selectin to the vascular sialomucin CD34. Science 262:436, 1993.

Beschorner WE, Civin CI, Strauss LC: Localization of hematopoietic progenitor cells in tissue with the anti-My-10 monoclonal antibody. Am J Pathol 119:1, 1985.

Bevilacqua MP, Nelson RM: Selectins. J Clin Invest 91:379, 1993.

Bollman JL, Cain JC, Grindlay JH: Techniques for the collection of lymph from the liver, small intestine, or thoracic duct of the rat. J Lab Clin Med 33:1349, 1948.

Carlos TM, Harlan, JM: Leukocyte-Endothelial adhesion molecules. Blood 84(7):2068, 1994

Chin Y-H, Sackstein R, Cai J-P: Lymphocyte-homing receptors and preferential migration pathways. Proc Soc Exp Biol Med 196:374, 1991.

Civin CI, Strauss SC, Brovall C, Fackler MJ, Schwartz JF, Shaper JH: Antigenic analysis of hematopoiesis III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells. J Immunol 133:157, 1984.

Cowing C, Chapdelaine JM: T cells discriminate between Ia antigens expressed on allogeneic accessory cells and B cells: a potential function for carbohydrate side chains on Ia molecules. Proc Natl Acad Sci USA 80:6000, 1983.

Cullen SE, Kindle CS, Shreffler DC, Cowing C: Differential glycosylation of murine B cell and spleen adherent cell Ia antigens. J Immunol 127:1478, 1981.

Gatenby PA, Kansas GS, Xian CY, Evans RL, Engleman EG: Dissection of immunoregulatory subpopulations of T lymphocytes within the helper and suppressor sublineages in man. J. Immunol 129:1997, 1982.

Gowans, JL, Knight EJ: The route of re-circulation of lymphocytes in the rat. Proc Roy Soc Lond B 159:257, 1964.

Griffin JD, Spertinin O, Ernst TJ, Belvin MP, Levine HB, Kanakura Y, Tedder TF: Granulocyte-macrophage colony-stimulating factor and other cytokines regulate surface expression of the leukocyte adhesion molecule-1 on human neutrophils, monocytes, and their precursors. J Immunol 145:576, 1990.

Huff TF, Uede T, Iwata M, Ishizaka K: Modulation of the biologic activities of IgE-binding factors. III. switching of a T cell hybrid clone from the formation of IgE-suppressive factor to the formation of IgE-potentiating factor. J Immunol 131:1090, 1983.

Imai Y, Lasky LA, Rosen SD: Sulphation requirement for GlyCAM-1, an endothelial ligand for L-selectin. Nature 361:555, 1993.

Imai Y, Singer MS, Fennie C. Lasky LA, Rosen SD: Identification of a carbohydrate-based endothelial ligand for a lymphocyte homing receptor. J Cell Biol 113:1213, 1991.

Kobayashi M, Imamura M, Uede T, Sakurada K, Maeda S, Iwasaki H, Tsuda Y, Musashi M, Miyazaki T: Expression of adhesion molecules on human hematopoietic progenitor cells at different maturational stages. Stem Cells 12:316, 1994.

Koeffler HP, Billing R, Lusis AJ, Sparkes R, Golde DW: An undifferentiated variant derived from the human acute myelogenous leukemia cell line (KG-1). Blood 56:265, 1980.

Krause DS, Fackler MJ, Smith OM, May WS: Two forms of CD34 protein are expressed in human KMT2 and KG1a cells. Blood 82:110a, 1993 (abstr)

Lasky LA, Singer MS, Dowbenko D, Imai Y, Henzel WJ, Grimley C, Fennie C, Gillet N, Watson SR, Rosen SD: An endothelial ligand for L-selectin is a novel mucin-like molecule. Cell 69:927, 1992.

Lewinsohn DM, Bagatze RR, Butcher EC: Leukocyte-endothelial cell recognition: evidence of a common molecular mechanism shared by neutrophils, lymphocytes, and other leukocytes. J Immunol 138:4313, 1987.

Majdic O, Stöckl J, Pickl WF, Bohuslav J, Strobl H, Scheinecker C, Stockinger H, Knapp W: Signaling and induction of enhanced cytoadhesiveness via the hematopoietic progenitor cell surface molecule CD34. Blood 83:1226, 1994.

Marchesi VT, Gowans JL: The migration of lymphocytes through the endothelium of venules in lymph nodes: an electron microscope study. Proc Roy Soc Lond B 159:283, 1964.

Ord DC, Ernst TJ, Shou LJ, Rambaldi A, Spertinin O, Griffin J, Tedder TF: Structure of the gene encoding the human leukocyte adhesion molecule-1 (TQ1, Leu-8) of lymphocytes and neutrophils. J Biol Chem 265:7760, 1990.

Oxley, SM, Sackstein, R: L-selectin-CD34 Interactions mediate functional binding of lymphocytes to hematopoetic progenator cells. Clin Res. Abs. 42(2):235A, April, 1994

Ramakrishnan S: Current status of antibody-toxin conjugates for tumor therapy. Targeted Diagn Ther 3:189, 1990.

Reinherz EL, Morimoto C, Fitzgerald KA, Hussey RE, Daley JF, Schlossman SF: Heterogeneity of human $T4^+$ inducer T cells defined by a monoclonal antibody that delineates two functional subpopulations. J Immunol 128:463, 1982.

Rosen SD, Singer MS, Yednock TA, Stoolman LM: Involvement of sialic acid on endothelial cells in organ-specific lymphocyte recirculation. Science 228:1005, 1985.

Rosen SD: Cell surface lectins in the immune system. Sem Imm 5:237, 1993.

Sackstein R: Physiological migration of lymphocytes to lymph nodes following bone marrow transplantation: Role in immune recovery. Seminars in Oncology 20(5), Suppl 6:34, 1993.

Sackstein R, Falanga V, Streilein JW, Chin YH: Lymphocyte adhesion to psoriatic dermal endothelium is mediated by a tissue-specific receptor/ligand interaction. J Invest Dermatol 91:423, 1988.

Selden RF: Transfection using DEAE-dextran, in Ausubel FM, Brent R, Kingston RE, Moor DD, Seidman JG, Smith JA, Struhl K (eds): Current Protocols in Molecular Biology, vol 2. New York NY, John Wiley & Sons, 1992, p. 9.2.1.

Sharon, N, Lis, H: Carbohydrates in cell recognition. Sci. Amer. 82-89, Jan., 1993.

Shaw S, Luce GEG, Quinones R, Gress RE, Springer TA, Sanders ME: Two antigen-independent adhesion pathways used by human cytotoxic T-cell clones. Nature 323:262, 1986.

Spertini O, Luscinskas FW, Kansas GS, Munro JM, Griffin JD, Gimbrone MA, Tedder TF: Leukocyte adhesion molecule-1 (LAM-1, L-selectin) interacts with an inducible endothelial cell ligand to support leukocyte adhesion. J Immunol 147:222565, 1991.

Spertinin O, Kansas GS, Reimann KA, Mackay CR, Tedder TF: Function and evolutionary conservation of distinct epitopes on the leukocyte adhesion molecule-1 (TQ1, Leu-i) that regulate leukocyte migration. J Immunol 147:942, 1991.

Springer TA, Dustin ML, Kishimoto TK, Marlin SD: The lymphocyte function-associated LFA-1, CD2, and LFA-3 molecules: Cell adhesion receptors of the immune system. Ann Rev Immunol 5:223-252, 1987.

Stamper HB, Woodruff JJ: Lymphocyte homing into lymph nodes: in vitro demonstration of the selective affinity of recirculating lymphocytes for high-endothelial venules. J Exp Med 144:828, 1976.

Stoolman LM, Rosen SD: Possible role for cell-surface carbohydrate-binding molecules in lymphocyte recirculation. J Cell Biol 96:722, 1983.

Stoolman LM, Tenforde TS, Rosen SD: Phosphomannosyl receptors may participate in the adhesive interaction between lymphocytes and high endothelial venules. J Cell Biol 99:1111535, 1984.

Streeter PR, Berg EL, Rouse BTN, Bargatze RF, Butcher EC: A tissue-specific endothelial cell molecule involved in lymphocyte homing. Nature 331:41, 1988.

Sutherland DR, Marsh JCW, Davidson J, Baker MA, Keating A, Mellors A: Differential sensitivity of CD34 epitopes to cleavage by *Pasteurella haemolytica* glycoprotease: Implications for purification of CD34-positive progenitor cells. Exp Hematol 20:590, 1992.

CLAIMS

What is claimed is:

1. An isolated and purified glycoprotein and functional analogs thereof characterized by

(a) being expressed on at least primitive hematopoietic cells,

(b) being a ligand for L-selectin, the binding of ligand to L-selectin not being inhibited by anti-CD34 antibodies; and

(c) not being identified by MECA-79 a monoclonal antibody which identifies ligands of L-selectin on lymph node high endothelial venules.

2. An isolated and purified glycoprotein as set forth in claim 1 wherein said glycoprotein is a membrane-associated glycoprotein.

3. An isolated and purified glycoprotein as set forth in claim 1 wherein said glycoprotein functions as an adhesion protein ligand.

4. An isolated and purified glycoprotein as set forth in claim 1 wherein said glycoprotein facilitates attachment of lymphocytes to hematopoietic cells.

5. At least one antibody directed against said glycoprotein as set forth in claim 1.

6. An antibody as set forth in claim 5 wherein said antibody is a monoclonal antibody.

7. A method of targeting cells expressing the glycoprotein as set forth in claim 1 including the steps of

preparing a monoclonal antibody directed against the glycoprotein as set forth in claim 1,

preparing an immunotoxin utilizng the antibody,

exposing a population of cells to said antibodies, and

killing cells bound to the immunotoxin.

Tedder TF, Penta AC, Levine HB, Freedman AS: Expression of the human leukocyte adhesion molecule, LAM1, identity with the TQ1 and Leu-8 differentiation antigens. J Immunol 144:532, 1990.

Terstappen LWMM, Huang S, Bui N, Picker LJ: Induction of human hematopoietic stem cell outgrowth by L-selectin ligation. Blood 82:109a, 1993 (abstr)

Terstappen LWMM, Huang S, Picker LJ: Flow cytometric assessment of human T-cell differentiation in thymus and bone marrow. Blood 79:666, 1992.

True EE, Singer MS, Lasky LA, Rosen SD: Requirement for sialic acid on the endothelial ligand of a lymphocyte homing receptor. J Cell Biol 111:2757, 1990.

Watson SR, Imai Y, Fennie C, Geoffroy JS, Rosen SD, Lasky LA: A homing receptor-IgG chimera as a probe for adhesive ligands of lymph node high endothelial venules. J Cell Biol 110:2221, 1990.

Woodruff JJ, Katz IM, Lucas LE, Stamper HB: An in vitro model of lymphocyte homing II. Membrane and cytoplasmic events involved in lymphocyte adherence to specialized high-endothelial venules of lymph nodes. J Immunol 119:1603, 1977.

Woodruff, JJ, Clark, LM, Chin, YH: Specific cell-adhesion mechanisms determining migration pathways of recirculating lymphocytes. Ann Rev Immunol 5:201-222, 1987.

Yamashita K, Hitoi A, Tateishi N, Higashi T, Sakamoto Y, Kobata A: Organ-specific difference in the sugar chains of gamma-glutamyltranspeptidase. Arch Biochem Biophys 225:993, 1983.

Yamashita K, Hitoi A, Tateishi N, Higashi T, Sakamoto Y, Kobata A: The structures of the carbohydrate moieties of mouse kidney gamma-glutamyltranspeptidase: occurrence of X-antigenic determinants and bisecting N-acetylglucosamine residues. Arch Biochem Biophys 240:573, 1985.

8. The method of claim 7 wherein the toxin is selected from the group consisting of ricin A chain, pseudomonas exotoxin A, diphtheria toxin and chemotherapeutic compounds.

9. The method of claim 7 wherein the cells are exposed to the immunotoxin *in vivo*.

10. The method of claim 7 further characterized by the cells being selected from the group consisting of leukemic cells, malignant hemopoietic progenitor cells and other malignant cells expressing the glycoprotein.

11. A method of selecting for cells expressing the glycoprotein as set forth in claim 1 including the steps of

preparing an antibody directed against the glycoprotein as set forth in claim 1,

exposing a population of cells to said antibodies, and

selecting cells bound to the antibody.

12. A method of selecting against cells expression the glycoprotein as set forth in claim 1 including the steps of

preparing an antibody directed against the glycoprotein as set forth in claim 1,

exposing a population of cells to said antibodies, and

removing cells bound to the antibody.

13. The method of claim 12 wherein said removing step is selected from complement-mediated lysis, panning, cell sorting.

14. A method of regulating hematopoiesis including the steps of

selecting cells with an appropriate level of expression of the glycoprotein as set forth in claim 1 from a patient,

culturing the selected cells, and

reinfusing the patient with the expanded selected cell population.

15. A method of regulating inflammatory response by interrupting cellular migration into lymph nodes and sites of chronic inflammation including the step of administering to a patient antibody directed against the glycoprotein as set forth in claim 1.

16. The method of claim 15 further characterized by the inflammatory response being as found in the group selected from autoimmune disorders, post-ischemic tissue injury and sepsis.

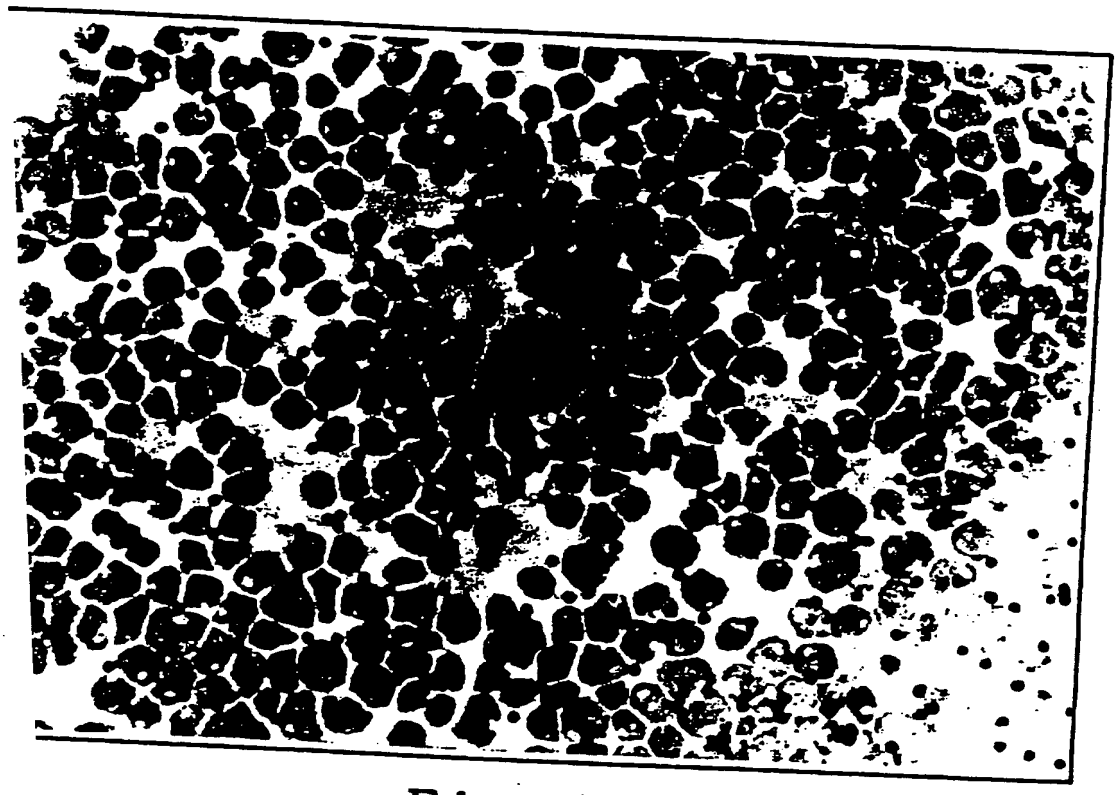

Fig-1A

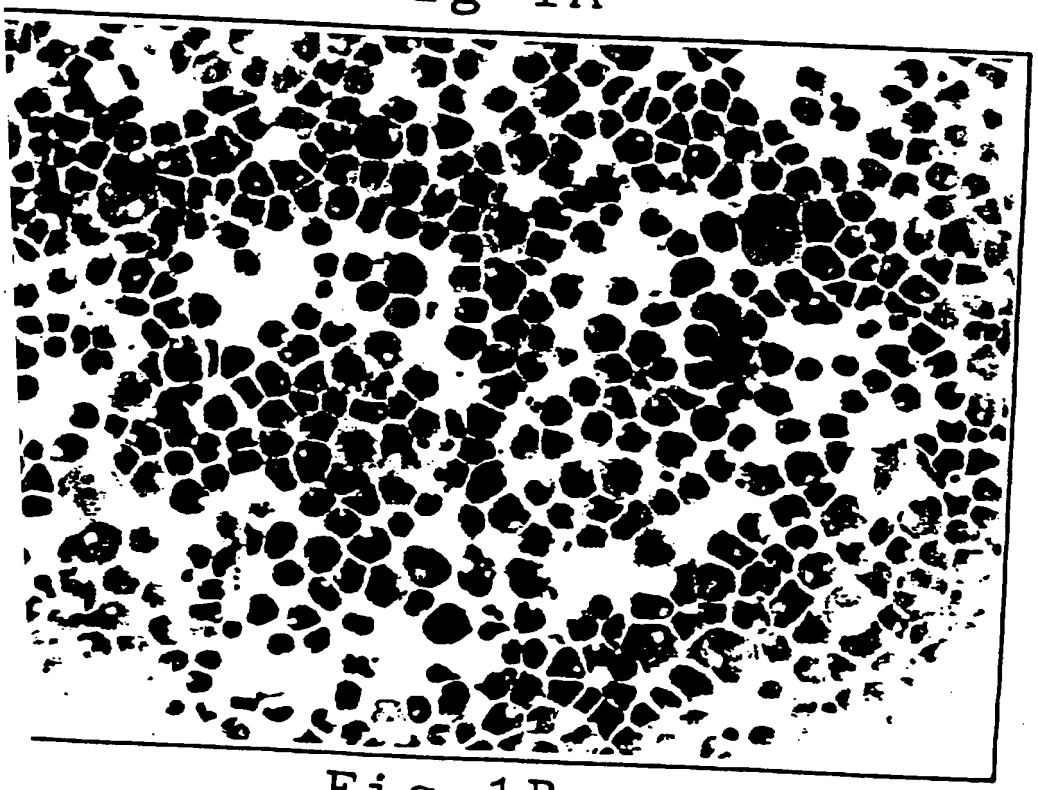

Fig-1B

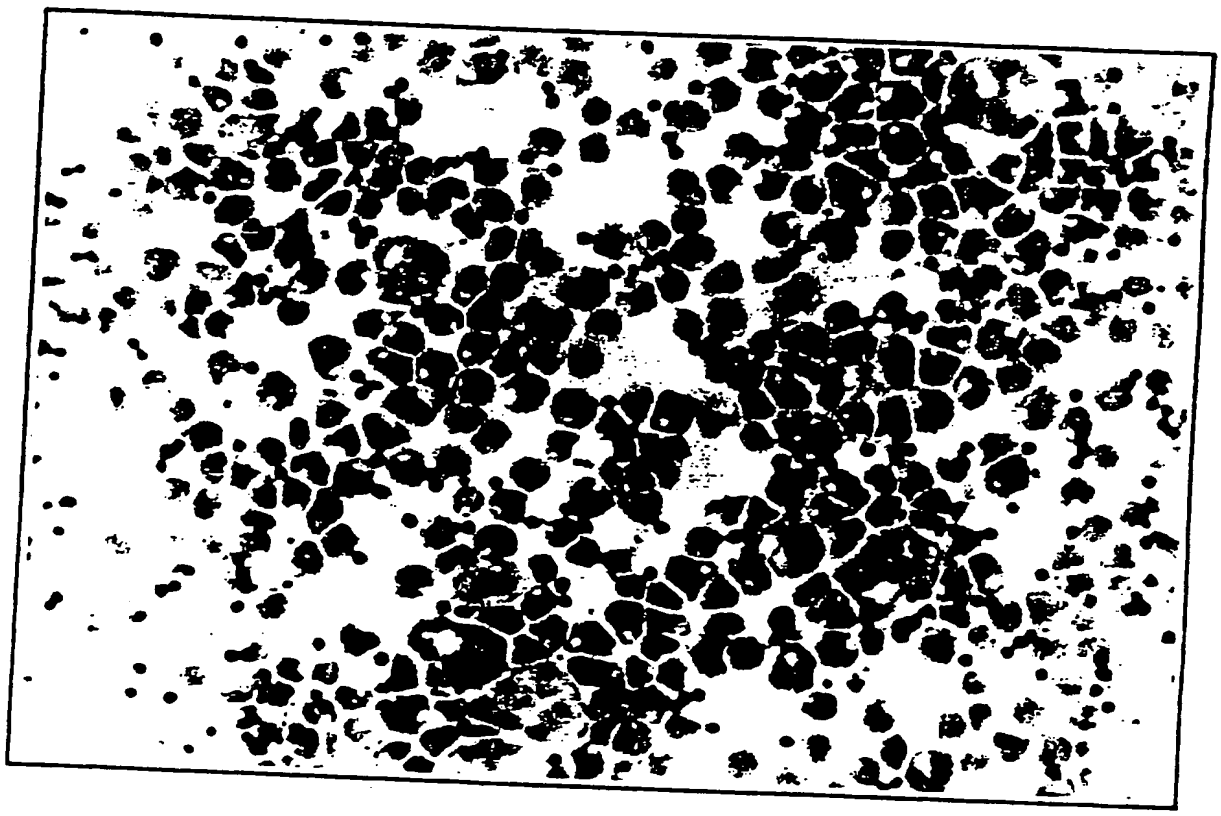

Fig-4

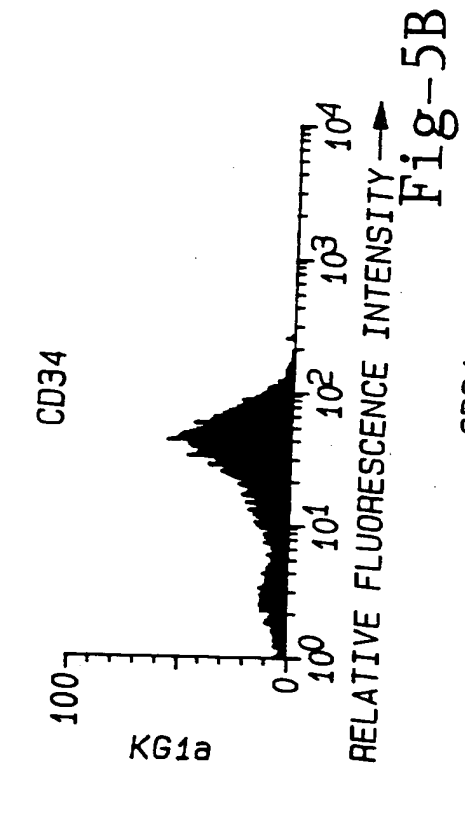

Fig-5B

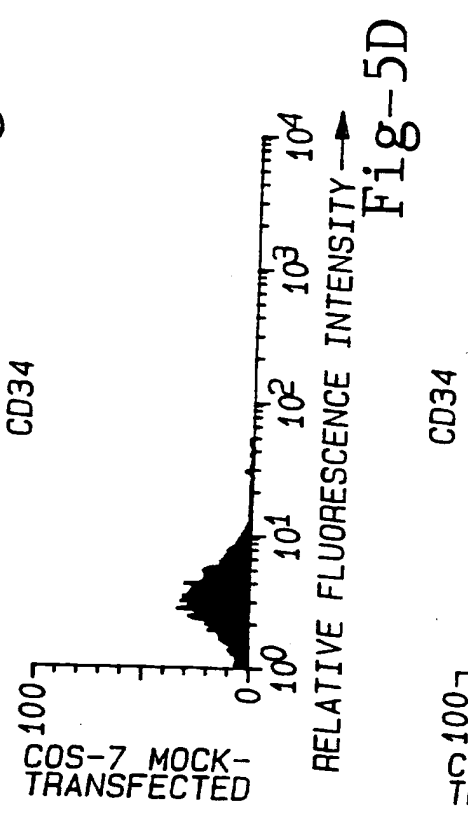

Fig-5D

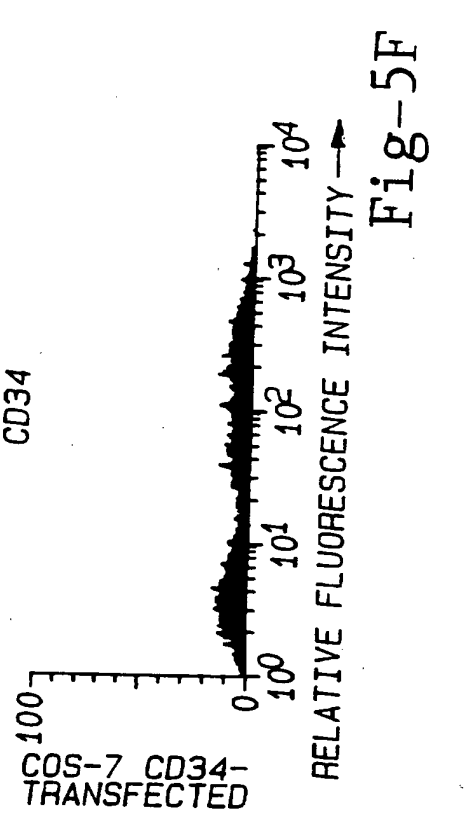

Fig-5F

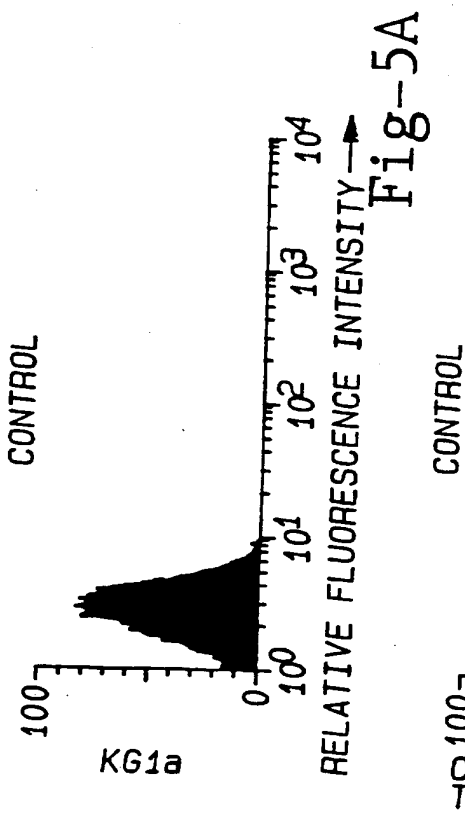

Fig-5A

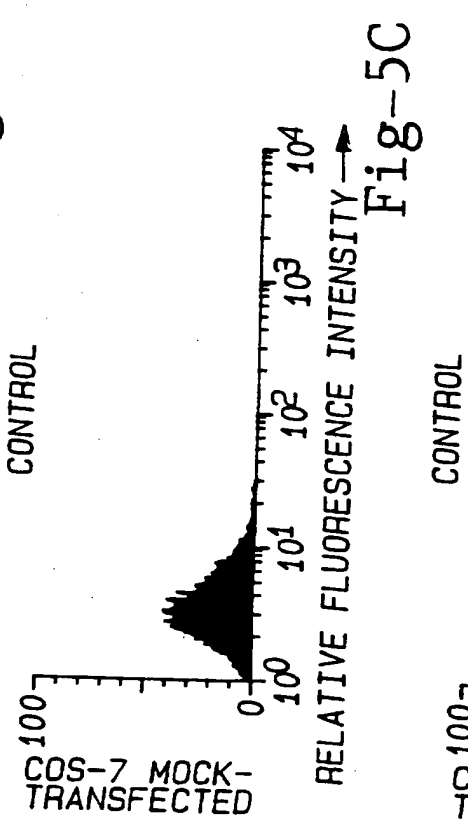

Fig-5C

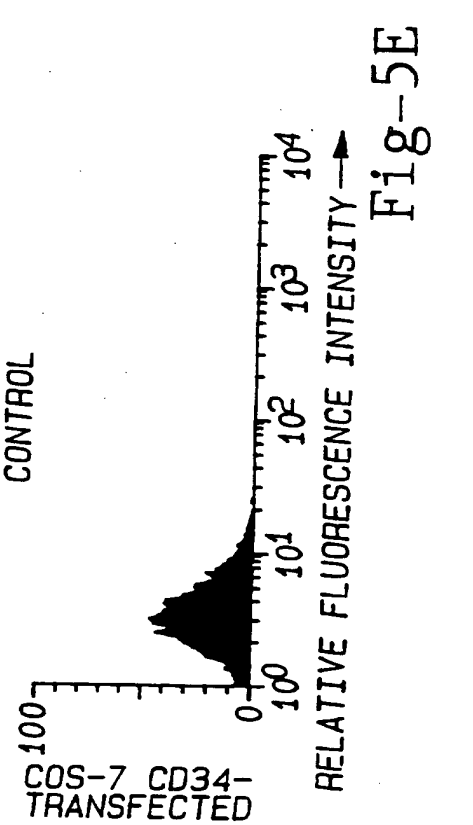

Fig-5E

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/13736

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/1.49, 93.7, 93.71, 140.1, 143.1, 153.1, 173.1; 530/350, 388.22, 388.7, 389.6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, BIOSIS, CA, EMBASE, MEDLINE, WPI
search terms: L-selectin, CD34, HLL, KG1a

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Experimental Hematology, Volumn 22, issued 1994, SACKSTEIN, "L-Selectin Mediates Lymphocyte Adhesion To KG1A Cells By binding To A Ligand Other Than CD34", page 788, see Abstract 414.	1-16
Y	Hematopoietic Cell Proliferation And Differentiation II, Volume 82, issued 1993, KRAUSE ET AL., "Two Forms Of CD34 Protein Are Expressed In Human KMT2 And KG1a Cells", page 110a, see Abstract 427.	1-16
Y	Histochemistry, Volumn 100, issued 1993, ROSEN, "L-Selectin And Its Biological Ligands", pages 185-191, see entire document.	1-16

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
17 JANUARY 1996

Date of mailing of the international search report
06 FEB 1996

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
PHILLIP GAMBEL
Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/13736

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Science, Volume 262, issued 15 October 1993, BAUMHUETER ET AL., "Binding Of L-Selectin To The Vascular Sialomucin CD34", pages 436-438, see entire document.	1-16
Y	US, A, 5,130,144 (CIVIN) 14 July 1992, see entire document.	1-16

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/13736

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 35/12, 35/14, 35/28, 39/395, 39/44; C07K 14/435, 14/705, 16/18, 16/28

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/1.49, 93.7, 93.71, 140.1, 143.1, 153.1, 173.1; 530/350, 388.22, 388.7, 389.6